Nonideal Effects of Protein Aggregation/Protein Activity on the Sorption of Water Vapor by Proteins: A Thermodynamic Linkage Study

1. INTRODUCTION

Sorption isotherms of water in foods are of considerable practical interest and have been the subject of various thermodynamic and mathematical treatments (Iglesias and Chirife, 1982; Baianu, 1992). Sigmoidal, or type II, water sorption isotherms were generally observed in food proteins and other systems. The relationship between the relative vapor pressure of water (RVP), or "water activity" ($\alpha_w = p/p_0$), and the moisture content M is often expressed by equations that can be utilized for *linear* regression analysis of the data [see, for example, van der Berg and Bruin (1981), Iglesias and Chirife (1982), and Asbi and Baianu (1986)]. (In the sequel, p is the vapor pressure of water in the hydrated protein system at equilibrium and p_0 is the vapor pressure of pure water.) A notable exception is the *five-parameter* model/*nonlinear* regression analysis of water sorption isotherms introduced by D'Arcy and Watt (1970, 1981) for food proteins and other systems. The model of D'Arcy and Watt (1970, 1981) involves binding of individual water molecules to *heterogeneous sites* of *two* types: strong and weak. Furthermore, at high α_w, the model postulates some "residual" binding of water "multilayers" to the macromolecular surface. This isodesmic model further assumes that *all* types of binding sites, including the multilayer ones, are being simultaneously filled (to different degrees) as the moisture content increases from very low to high levels. Thus, the multilayer water would be present (in small amounts) even at very low α_w, according to this model. The proof that the D'Arcy and Watt model is indeed an isodesmic one is given in the Appendix, where a complete derivation of the D'Arcy and Watt theoretical sorption isotherm is given in terms of water binding equilibria. The implicit assumptions and approximations (D'Arcy and Watt, 1970) involved in the latter model are also pointed out in the Appendix, through this derivation. As shown previously (Asbi and Baianu, 1986), the D'Arcy and Watt model does *not* fit very well experimental sorption isotherms at high α_w ($\gtrsim$0.93) because of the multilayer assumption; the linearized version of the model that does *not* assume the presence of multilayers of water (Asbi and Baianu, 1986) fits quite well the experimental data up to very high α_w (*in the absence of any added salt*).

On the other hand, parallel measurements of sorption isotherms and nuclear magnetic relaxation rates of water in food proteins (with and without added salts) were recently reported (Lioutas et al., 1986, 1987, 1988; Myers-Betts and Baianu, 1990), which strongly suggest novel water sorption mechanisms in foods. Such NMR results over a wide range of hydration levels of lysozyme, myofibrillar proteins [Figures 1 and 3 of Lioutas et al. (1988)], corn zeins [Figures 4–6 of Myers-Betts and Baianu (1990)], and soybean proteins showed that the NMR transverse relaxation rate of water (that measures the average molecular mobility of water) changes little at high protein concentrations ($\gtrsim$65% w/w), whereas α_w decreases rapidly. Since the NMR transverse relaxation rate of water is an average over the "free" (or bulk) and "bound" water populations, these surprising results strongly indicate that the interpretation of water activity in foods strictly in terms of only bound and free water populations is basically naive. Furthermore, at the lower protein concentrations ($C < 0.3$ g of protein/g_{total}), in the absence of added salt and near neutral pH, α_w was nearly constant at about 0.99, whereas the NMR transverse relaxation rate (R_2) of water changed rapidly and nonlinearly with concentration. In a series of recent papers it was shown that in this concentration range the R_2 values of water depended not only on the *hydration* of the proteins but also on the *protein–protein interactions*, or *protein activity* (Kumosinski and Pessen, 1982; Kakalis and Baianu, 1988; Lioutas et al., 1988; Myers-Betts and Baianu, 1989; Baianu et al., 1990). At the lower protein concentrations ($C \lesssim 0.2$ g of protein/g_{total}), the protein charge–charge interactions had the largest contributions to the protein activity (Kumosinski and Pessen, 1982; Myers-Betts and Baianu, 1989, 1990), whereas at the higher protein concentrations *protein aggregation* dominated protein activity. It was, therefore, proposed that the relative vapor pressure of water in food proteins is markedly reduced by *aggregated protein* molecules at protein concentrations higher than about 65% w/w (Lioutas et al., 1988; Myers-Betts and Baianu, 1990).

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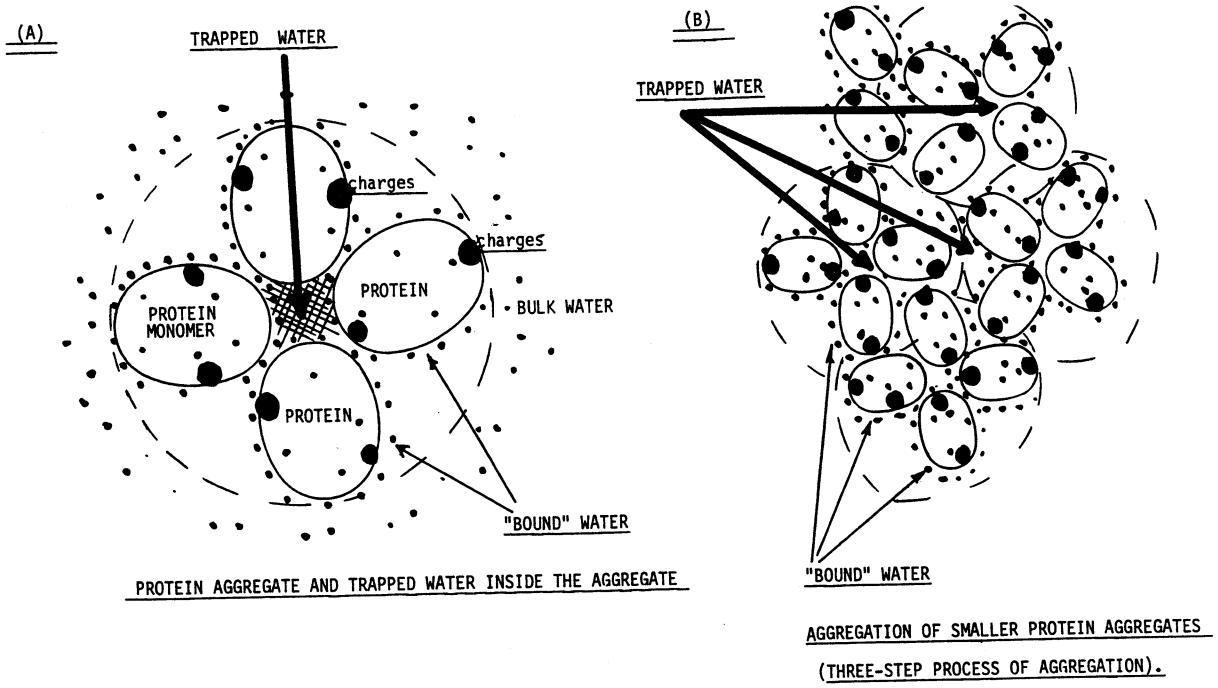

Figure 1. Schematic representation of the protein aggregation process described by eqs 1 and 2. Water trapped between the protein molecules in the aggregate is distinct from bound water.

We will present an attempt to model water sorption isotherms of food proteins by taking into account the entrapment of water caused by protein aggregation, as well as other protein–protein interactions (Figure 1). At the beginning, the number of our model parameters will be limited to *five* so that the results obtained by nonlinear regression analysis with our protein aggregation/activity model can be directly compared with those obtained with the *five-parameter, isodesmic* model of D'Arcy and Watt (1981). Whereas the D'Arcy–Watt isodesmic model assumes *four* water populations ["strongly bound", "weakly bound", "multilayer", and free (bulk) water], our protein activity/aggregation model postulates only *three* water populations: bound water, trapped water, and free (bulk) water. The latter three water populations can be monitored by nuclear magnetic relaxation measurements (and other NMR techniques, as well) and were found previously to exhibit distinct dynamic/motional and relaxation properties. The distinction between bound and trapped water could be practically made either by pulsed gradient NMR or by the property of most trapped water of being *freezable* at low temperatures (e.g., below about −50 °C), whereas the *bound* water would *not* be freezable at such low temperatures.

It is interesting that amino acids such as glycine and L-glutamic acid (Edsall, 1958), as well as the imino acid proline, sarcosine, and glycine–betaine (Lilley and Sutton, 1991), exhibit also **nonideal** behaviors in their water sorption isotherms. The relative vapor pressure of water measured at equilibrium for systems of these concentrated solutes is *substantially* **lower** than the α_w value predicted by an **ideal** model, even with **cooperative** hydration interaction between the amino or imino acids [Table 6 of Lilley and Sutton (1991)]. The explanation given by Lilley and Sutton (1991) in the latter case involves solute–solute interactions (or solute activity); this is consistent with the protein activity/interaction effects on water sorption that are here considered.

2. THEORY

We consider the following protein aggregation and water entrapment scheme (Figure 1 and eqs 1 and 2)

$$nP + qW \overset{k_1^n}{\rightleftarrows} P_nW_q \tag{1}$$

$$m(P_2W_q) + rW \overset{k_2^m}{\rightleftarrows} (P_nW_q)_mW_r \tag{2}$$

where P is the hydrated monomeric protein, W is the water, k_1^n and k_2^m are association constants, and n, q, m, and r are mol numbers of the various species. Only protein-associated water in excess of that of the monomer P is indicated in eqs 1 and 2. The observed decrease in α_w (or p/p_0) with increasing protein concentration is attributed to the entrapment of water within protein aggregates (Figure 1).

The observed water activity α_w can be expressed as a linear combination of water activity contributions from the different protein species present

$$\alpha_w = f(W) + \alpha_{w1} f(P_nW_q) + (\alpha_{w2} - \alpha_{w1}) f((P_nW_q)_mW_r) \tag{3}$$

where α_{w1} and α_{w2} are the contributions to the water activities due to the protein species P_nW_q and $(P_nW_q)_mW_r$, respectively (that of free water, W, is 1.00), and $f(P_nW_q)$ and $f((P_nW_q)mW_r)$ are the fractions of the water (on a molar basis) bound and trapped by the corresponding protein species. It should be stressed that eq 2 implies sequential equilibria, i.e., $k_1 > k_2$, and also that the formation of P_nW_q is completed before the formation of $(P_nW_q)_mW_r$ begins. In view of the high total protein concentrations encountered in sorption studies (typically 3–30 g of protein/g of water or more) it is reasonable to assume that the amount of protein monomer is negligible when compared to that of the aggregated protein; therefore, there is only a negligible contribution from the hydrated protein monomer P in eq 3. By definition

$$f(W) = \frac{[W]}{[W] + q[P_nW_q]} = \frac{[W]}{[W] + qk_1^n[P]^n[W]^q} \quad (4)$$

$$f(P_nW_q) = \frac{q[P_nW_q]}{[W] + q[P_nW_q]} = \frac{qk_1^n[P]^n[W]^q}{[W] + qk_1^n[P]^n[W]^q} \quad (5)$$

If water binding is noncooperative, then we can set $q = 1$ in eqs 4 and 5. The reason is that binding data for identical independent binding sites is indistinguishable from ligand binding to a single site (Hammes, 1982). Then

$$f(W) = 1/(1 + K_1^n[P]^n) \quad (6)$$

$$f(P_nW_q) = K_1^n[P]^n/(1 + K_1^n[P]^n) \quad (7)$$

Similarly

$$f((P_nW_q)_mW_r) = \frac{(M+1)k_2^m[P]_n[W_q]^m}{1 + (m+1)k_2^m[P]_n[W_q]^m} \quad (8)$$

The total protein concentration C is generally

$$C = [P] + n[P_nW_q] + nm[(P_nW_q)_mW_r] \quad (9)$$

During the first aggregation step there is no $(P_nW_q)_mW_r$ present and

$$C = [P] + n[P_nW_q] = [P](1 + nk_1^n[P]^{n-1}[W_q]) \quad (10)$$

The second term in parentheses is much smaller than unity and can be discarded. The reason is the low molecular protein concentration that results from the large protein molecular weights (see also section 5).

Then

$$C = [P] \quad (11)$$

and

$$f(W) = 1/(1 + k_1^nC^n) \quad (12)$$

$$f(F_nW_q) = k_1^nC^n/(1 + k_1^nC^n) \quad (13)$$

During the second protein aggregation step, there is little protein monomer present and

$$C = n[P_nW_q] + nm[(P_nW_q)_mW_r] \quad (14)$$

(if $k_1 < 1.0$) or

$$C = n[P_nW_q](1 + mk_2^m[P_nW_q]^{m-1}[W]^r) \quad (15)$$

In this case, after a similar approximation

$$C = n[P_nW_q] \quad (16)$$

and

$$f((P_nW_q)_mW_r) = \frac{(m+1)n^{-m}k_2^mC^m}{1 + (m+1)n^{-m}k_2^mC^m} \quad (17)$$

Taking into account eqs 12, 13, and 17, we can rewrite eq 3 expressing the total protein concentration C in grams of protein/gram of water (which is being used experimentally) rather than moles/liter. After the various constant and conversion factors ($K = (1000M_w)k$) are grouped together

$$\alpha_w = \frac{1}{1 + K_1^nC^n} + \alpha_{w1}\frac{K_1^nC^n}{1 + K_1^nC^n} + (\alpha_{w2} - \alpha_{w1})\frac{K_2^mC^m}{1 + K_2^mC^m} \quad (18)$$

The K's here are parameters related to the initial equilibrium constants K_i; n and m are the degrees of aggregation for the cooperative protein aggregation. In the case of an additional third aggregation reaction

$$s(P_nW_q)_mW_r + tW \overset{k_3}{\rightleftarrows} ((P_nW_q)_mW_r)_sW_t \quad (19)$$

that is now considered to be nonsequential, i.e., there is simultaneous formation of $(P_nW_q)_mW_r$ and $(P_nW_q)_m$-$W_r)_sW_t$, one needs to modify eq 18 accordingly

$$\alpha_w = f(W) + \alpha_{w1}f(P_nW_q) + (\alpha_{w2} - \alpha_{w1})f((P_nW_q)_mW_r) + (\alpha_{w3} - \alpha_{w1})f(((P_nW_q)_mW_r)_sW_t) \quad (20)$$

that is, a mixture of secondary aggregates and "simple" primary aggregates. If the fraction of the total aggregate of the form P_nW_q is $K_2^mC^m/(1 + K_2^mC^m)$, then, with procedures analogous to those used to obtain eqs 13 and 17, after suitable substitutions, one may write

$$f(((P_nW_q)_mW_r)_sW_t) = \frac{K_2^mC^m}{1 + K_2^mC^m}\frac{K_3^sC^s}{1 + K_3^sC^s} \quad (21)$$

and

$$f((P_nW_q)_mW_r) = \frac{K_2^mC^m}{1 + K_2^mC^m}\left(1 - \frac{K_3^sC^s}{(1 + K_3^sC^s)}\right) - \frac{K_2^mC^m}{1 + K_2^mC^m}\frac{1}{1 + K_3^sC^s} \quad (22)$$

Then

$$\alpha_w = \frac{1}{1 + K_1^nC^n} + \alpha_{w1}\frac{K_1^nC^n}{1 + K_1^nC^n} + (\alpha_{w2} - \alpha_{w1})\frac{K_2^mC^m}{1 + K_2^mC^m}\frac{1}{1 + K_3^sC^s} + (\alpha_{w3} - \alpha_{w1})\frac{K_2^mC^m}{1 + K_2^mC^m}\frac{K_3^sC^s}{1 + K_3^sC^s} \quad (23)$$

The K's in eq 23 are also apparent binding constant parameters; n, m, and s are again the degrees of aggregation for the corresponding cooperative protein aggregation "reactions". Equations 3, 18, and 23 do not apply, as stated, if the solvent contains solutes other than protein (e.g., salt); the solvent water activity is then less than 1.0 and its actual value must replace 1.0.

3. METHODS

In the present study we have made use of water sorption data for proteins that are available in the literature. The adsorption data at 40 °C for lyophilized hen egg white albumin, equine serum albumin, lyophilized β-lactoglobulin, two corn zein fractions, silk (fibroin), gelatin from hide collagen, bovine elastin, and salmin (all with α_w ranging from 0.050 to 0.950) were those tabulated by Bull (1944). D'Arcy and Watt's data at 35 °C for Merino wool (keratin) and skin collagen (D'Arcy and Watt, 1970) were obtained from an enlargement of their adsorption isotherm (Figure 1 in ref 9) using a coordinate digitizer interfaced to a Modcomp Classic minicomputer to maintain a high degree of precision (the exact numerical values of water activity and protein concentration were not reported). The α_w range is from 0.054 to 0.960 (keratin) and from 0.051 to 0.937 (collagen). Adsorption isotherm data at 20 °C for hen egg white lysozyme (Lioutas et al., 1987) and bovine myofibrillar proteins in the absence of salt or with 4% NaCl (Lioutas et al., 1988) have been tabulated.

These three data sets are particularly noteworthy because they cover a wide water activity range (0.005–0.997 for lysozyme, 0.113–0.996 for myofibrillar proteins without salt, and 0.113–0.965 for myofibrillar proteins with salt).

Table I. Results of Nonlinear Regression Analysis of M (Grams of Water/Gram of Protein) vs α_w Data for Various Proteins onto Equation A14, Representing the Isodesmic Model of D'Arcy and Watt (1981)

	parameters				
protein	K_1[a]	K_1'	B	K_2[a]	K_2'
egg albumin	13.00 ± 5.43	0.041 ± 0.009	0.083 ± 0.018	0.936 ± 0.012	0.022 ± 0.003
serum albumin	3.86 ± 2.21	0.136 ± 0.077	−0.026 ± 0.085	0.908 ± 0.027	0.041 ± 0.014
β-lactoglobulin	19.25 ± 12.85	0.026 ± 0.007	0.117 ± 0.115	0.949 ± 0.009	0.021 ± 0.003
zein B	22.03 ± 14.52	0.024 ± 0.006	0.065 ± 0.013	0.950 ± 0.017	0.011 ± 0.003
zein C	17.69 ± 20.27	0.026 ± 0.011	0.061 ± 0.020	0.946 ± 0.019	0.013 ± 0.004
fibroin	13.06 ± 3.89	0.030 ± 0.005	0.065 ± 0.008	0.963 ± 0.007	0.011 ± 0.001
gelatin	7.75 ± 3.75	0.115 ± 0.038	0.057 ± 0.054	0.955 ± 0.014	0.034 ± 0.008
elastin	283.0 ± 1160.6	0.021 ± 0.005	0.164 ± 0.011	0.996 ± 0.009	0.007 ± 0.001
salmin	387.4 ± 4011.7	0.037 ± 0.016	0.007 ± 0.005	0.936 ± 0.008	0.119 ± 0.013
keratin	24.24 ± 8.10	0.031 ± 0.004	0.136 ± 0.009	0.917 ± 0.009	0.020 ± 0.002
collagen	3.24 ± 1.93	0.278 ± 0.218	−0.596 ± 0.512	0.584 ± 0.110	0.596 ± 0.512
lysozyme	84.2 ± 26377.0	0.036 ± 2.437	0.109 ± 3.243	1.000 ± 0.006	0.011 ± 0.021
myofibrillar	0.24 ± 255.6	4.660 ± 8658.2	−0.079 ± 908.0	0.985 ± 0.190	0.028 ± 0.366
myofibrillar, salt	3.33 ± 2102.6	0.130 ± 71.947	0.041 ± 50.611	0.998 ± 0.872	0.041 ± 1.254

[a] The association constants K_1 and K_2 are given in (g of water/g of protein)$^{-1}$.

Nonlinear regression analysis of water vapor sorption isotherms was performed using a program in FORTRAN based on a well-tested Gauss–Newton algorithm (Motulsky and Ransnas, 1987) and run on a Modcomp Classic minicomputer. Equation 24 is used for the protein aggregation model and can be recast in the following form with fewer parameters (there are only *five* for the simple aggregation model):

$$\alpha_w = \frac{1}{1+(K_1C)^n} + A_1\frac{(K_1C)^n}{1+(K_1C)^n} + A_2\frac{(K_2C)^m}{1+(K_2C)^m}\frac{1}{1+(K_3C)^s} + A_3\frac{(K_2C)^m}{1+(K_2C)^m}\frac{(K_3C)^s}{1+(K_3C)^s} \quad (24)$$

In most cases, the addition of an A_4C term to eq 24 improved noticeably the fitting at the higher protein concentrations. Such a term may arise from the fact that the protein powder used for sample preparation was not completely dry. Data were analyzed by minimizing the sum of squares of the residuals (SSR) and the root mean square (RMS) iteratively for a given set of n, m, and s values, thus obtaining the best-fit values for the A and K parameters. Then n, m, and s were fixed to new integer values, one at a time, and the A and K parameters were reiterated. The reported values are those that yielded the overall lowest RMS value. In the case of the protein aggregation model, both five-parameter (A_1, A_2, K_1, K_2, A_4) and seven-parameter (A_1, A_2, A_3, K_1, K_2, K_3, A_4) fittings were attempted that correspond to two-step and three-step aggregation schemes, respectively.

For the various comparisons of the goodness of fit for a given model, the F test was used with the F value

$$F = \frac{(\mathrm{SSR}_1 - \mathrm{SSR}_2)/(d_{f1} - d_{f2})}{\mathrm{SSR}_2/d_{f2}} \quad (25)$$

where SSR refers to the sum of squares of the residuals and d_f to the number of degrees of freedom (number of data points minus number of fitting parameters). The subscript "1" refers to the simpler model (the one with the fewer parameters). F distribution tables were consulted for $(d_{f1} - d_{f2})$ degrees of freedom at 25, 10, 5, 2.5, 1, 0.5, and 0.1% levels of significance (Snedecor, 1956; Beyer, 1984).

No direct comparison of the goodness of fit between the two different models discussed here is possible due to the difference in the respective y scales: the y axis in the D'Arcy–Watt isodesmic model is moisture content (grams of water/gram of protein) with a range from 0.0 to infinity (eq A1), whereas in the protein aggregation model it is the water activity α_w, with a range from 0.0 to 1.1 (eq 24). To make such a comparison possible, we calculated for every data set and for each model a normalized SSR value

$$\mathrm{SSR}_{norm} = \sum\left(\frac{y_{exp} - y_{calc2}}{y_{exp}}\right) \quad (26)$$

that was used in eq 25.

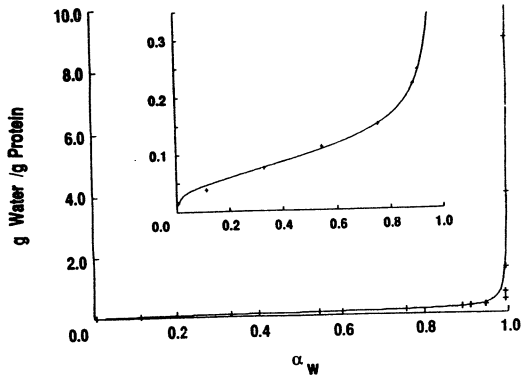

Figure 2. Sorption data of lysozyme analyzed according to the D'Arcy–Watt isodesmic model. The isotherm was calculated from eq (A1) using the fitting parameters of Table I. The deviations at α_w near 1.0 are marked. The inset shows the lower α_w data points of the same data set and the familiar sigmoidal theoretical isotherm (eq A1).

In the case of the myofibrillar proteins–NaCl system the water activity of 4% NaCl solution is 0.965 (Lioutas et al., 1988); the first term in eqs 3 and 20 was multiplied by this value.

4. RESULTS

D'Arcy and Watt Isodesmic Model. The results of our nonlinear regression analysis of the protein data sets with eq A14 are given in Table I. Our fittings with eq A14 are generally better than the ones originally reported (D'Arcy and Watt, 1970, 1981); however, the analyses for lysozyme (Figure 2) and the two myofibrillar protein samples with eq A14 are characterized by large variations in the estimated D'Arcy–Watt parameters (Table I) and high SSR values (Table II). We believe that the main reason for these variations is the breakdown of the D'Arcy and Watt model at high α_w values when protein dissolution and swelling in the sorbed water begin above about α_w = 0.95.

Negative B values do not have any physical meaning in the D'Arcy and Watt model (eq A14); in the case of serum albumin and collagen (Table I), B values are either negative or close to zero, and this fact seems to indicate that the D'Arcy and Watt model does *not* fit well these protein water sorption isotherms. The unusually high K_1 values for elastin and salmin are probably the result of experimental errors at low α_w. As previously noted (D'Arcy and Watt, 1970), the isotherms do not appear to pass through the origin.

For the other proteins the small variation in the values of the binding constants K_1 and K_2 (Table I) suggests a

Table II. Assessment of the Goodness of Fit for Protein Sorption onto Equation A14 for the D'Arcy and Watt Model

protein	SSR values[a] for parameters of D'Arcy and Watt (1970)[b]	for parameters of D'Arcy and Watt (1981)[c]	for parameters of Table I, present study
egg albumin	0.273×10^{-4}	0.291×10^{-4}	0.183×10^{-4}
serum albumin	0.157×10^{-3}	0.939×10^{-4}	0.630×10^{-4}
β-lactoglobulin	0.374×10^{-4}	0.386×10^{-4}	0.226×10^{-4}
zein B	0.364×10^{-4}	0.926×10^{-2} [e]	0.189×10^{-4}
zein C	0.245×10^{-2}	0.243×10^{-2}	0.155×10^{-4}
fibroin	0.105×10^{-4}	0.868×10^{-5}	0.572×10^{-5}
gelatin	0.677×10^{-3}	0.221×10^{-3}	0.131×10^{-3}
elastin[g]	0.468×10^{-4}	0.292×10^{-4}	0.336×10^{-4}
salmin	0.425×10^{-3}	0.481×10^{-3} [f]	0.405×10^{-3}
keratin	0.590×10^{-3}	0.601×10^{-3}	0.771×10^{-5}
collagen	0.692×10^{-3}	0.627×10^{-3}	0.324×10^{-4}
lysozyme	NA[d]	NA[d]	46.27
myofibrillar	NA[d]	NA[d]	67.04
myofibrillar, salt	NA[d]	NA[d]	70.11

[a] Sum of squares of the residuals, sum $[(M_{exp} - M_{calc})^2]$; M_{calc} obtained from eq 1. [b] Use of eq 1 with parameters from Table I of the reference; obtained without iteration. [c] Use of eq 1 with parameters from Table III of the reference; obtained without iteration. [d] NA, not available. [e] The calculated curve deviates considerably from the data points at high α_w. The reasons are not clear but a (typing) error in a fitting parameter value may be responsible. [f] No C value (B in eq 1) was given in Table III of D'Arcy and Watt (1981); a zero value was assumed, in agreement with Table I of D'Arcy and Watt (1970). [g] There is no statistical difference (F test, 25% level) between the fittings that correspond to the second- and third-column SSR values.

similarity for the water binding sites throughout this diverse group of proteins. "Tight" water binding (higher K_1 values) by presumably protein-ionized groups (Kuntz and Kauzmann, 1974) is approximately an order of magnitude stronger than the water binding involved in multilayer formation (K_2 values). No comparison of the K_1 and K_2 values obtained here with values determined by other techniques is possible because of the absence of data on the energetics of water binding to proteins (Kuntz and Kauzmann, 1974).

For the same data, our nonlinear regression analysis results with eq A14 (Table I) differ from those of D'Arcy and Watt (1970, Table I). This is most likely due to differences in the computation algorithms used. This may also be the reason for the different fitting parameters (eq A14) of the same data sets previously provided [D'Arcy and Watt (1970), Table 1, vs D'Arcy and Watt (1981), Table III]. Generally, we arrived at lower SSR values (Table II). There is no significant difference in the fittings at the 25% level for egg albumin, β-lactoglobulin, zein C, elastin, and salmin. Our fittings for serum albumin, zein B, and fibroin are better at the 25% level than for gelatin at the 5% level or for collagen at the 0.5% level, and our fitting for keratin is significantly better at the 0.1% level.

Protein Aggregation Model. The nonlinear regression analysis results of the protein sorption data according to the protein aggregation model are given in Tables III–V. Typical fittings for a variety of proteins are presented in Figures 3 and 4. Unlike the isodesmic model, the protein aggregation model holds well even at high α_w (Figure 3).

We have considered both two- and three-step aggregation for all data sets. From the A values (Tables III and IV) one may calculate the water activity contributions of the different protein species. By definition, α_w values should be between 0.0 and 1.0. In the case of β-lactoglobulin, fibroin, salmin, keratin, and collagen, only the three-step aggregation model (eq 23) yielded such meaningful α_w values. We have been able to fit the serum albumin, zeins, elastin, and lysozyme data sets only by means of the two-step aggregation model (eq 18). The three-step aggregation improved significantly the fittings for egg albumin, gelatin, and myofibrillar proteins with salt.

Note that negative values of $A_2 = (\alpha_{w2} - \alpha_{w1})$ were obtained (Table III) for most of the proteins considered; such values are the result of $\alpha_{w2} < \alpha_{w1}$, which is fully consistent with our protein activity/aggregation and water entrapment model (Figure 1 and eqs 1 and 2). The water population associated with protein aggregates has α_{w2} values which are lower than the α_{W1} values for the water bound to the protein monomers. Furthermore, the larger and more tightly packed the protein aggregates, the lower would be the value of α_{w2} for water associated with such large aggregates. The exception to the negative values of A_2 is provided by gelatin that already contains aggregated proteins instead of monomers since it gels readily through hydrogen bonding.

5. DISCUSSION

A comparison between the aggregation and the isodesmic models using the F test with the SSR_{norm} values of Table VI shows that the aggregation model is superior to the D'Arcy and Watt (isodesmic) one for egg albumin, serum albumin, β-lactoglobulin, zein B, fibroin, salmin, myofibrillar proteins (with or without salt), lysozyme (all at 0.1% level), collagen (0.5% level), and elastin and keratin (25% level). In the case of zein C there is no significant difference between the two models down to the 25% level of statistical significance.

If a theoretical curve is appropriate for a data set, the distribution of positive and negative residuals is random. In the case of **systematic differences**, residuals of the same sign tend to cluster together at different parts of a residual plot. As a result, the number of series of consecutive points with a residual of the same sign (number of runs) will be smaller (Motulsky and Ransnas, 1987). The number of runs for the fits of protein sorption data is consistently smaller in the case of the D'Arcy and Watt isodesmic model (data not shown), suggesting that the protein aggregation model is more appropriate for the description of water vapor sorption by proteins.

The accuracy of approximations made in the derivation of eq 18 can now be assessed from the calculated parameters (Table III). It has been assumed that $nK_1^n[P]^{n-1}[w]^q \ll 1$ (eq 10) and $mK_2^m[P_nW_q]^{m-1}[W]^r$ (eq 15), with $q = r = 1$. From Table III, using lysozyme as an example (monomer MW of 14 300) $n = 4$ and $m = 3$; also $K_1 = 0.157$ and $K_2 = 0.105$. Generally, from eqs 13, 17, and 18, $K_1^n = (xk_1)^n$ and $K_2^m = (m + 1)n^{-m}(xk_2)^m$, where x is the protein concentration conversion factor from moles/liter (C_M) to grams of protein/gram of water (C_g)

$$C_g = C_M(MW)/(1000 - C_M(MW)\bar{V}) \quad (27)$$

or

$$C_M = 1000C_g/(MW(1 + C_g\bar{V})) \quad (28)$$

$\bar{V}$ is the protein specific volume (0.69 mL/g for lysozyme), MW is the protein molecular weight, and the specific density of water is taken as 1.0 g/mL. For the most dilute sample studied (0.11 g of lysozyme/1.00 g of water at $\alpha_w = 0.997$) we are concerned with the first aggregation step only with $C_M = 7.1$ mM (eq 28) and $x = 15.38$ (eq 27). Then $k_1^n = 2.59 \times 10^{-7}$ and $nk_1^n[P]^{n-1}[W] = 2.17 \times 10^{-9} \ll 1$ for $[P] = 0.0071$ M and $[W] = 55.5$ M. Similarly, for the most

Table III. Results of Five-Parameter Nonlinear Regression Analysis of α_w vs C (Grams of Protein/Grams of Water) Data for Various Proteins According to Equation 25[a] for the Best n and m Values (Two-Step Protein Aggregation)

protein	parameters						
	A_1	K_1[b]	A_2	K_2[b]	n	m	A_4
egg albumin	0.216 ± 0.014	0.1132 ± 0.0012	−0.070 ± 0.016	0.0487 ± 0.0028	3	8	−0.00227 ± 0.00052
serum albumin	0.064 ± 0.039	0.1291 ± 0.0033	0.098 ± 0.037	0.1912 ± 0.0130	3	6	−0.00321 ± 0.00053
zein B	0.208 ± 0.022	0.0563 ± 0.0017	−0.169 ± 0.025	0.1236 ± 0.0045	3	5	−0.00007 ± 0.00040
zein C	0.201 ± 0.017	0.0604 ± 0.0015	−0.128 ± 0.020	0.1413 ± 0.0062	3	5	−0.00052 ± 0.00033
gelatin	0.002 ± 0.069	0.1814 ± 0.0076	0.158 ± 0.066	0.2695 ± 0.0197	3	6	−0.00491 ± 0.00143
elastin	0.661 ± 0.040	0.1084 ± 0.0060	−0.266 ± 0.043	0.1627 ± 0.0030	4	6	−0.01137 ± 0.00054
lysozyme[c]	0.924 ± 0.172	0.1575 ± 0.0353	−0.794 ± 0.167	0.1050 ± 0.0109	4	3	−0.00190 ± 0.00045
myofibrillar[c]	0.433 ± 0.023	0.1344 ± 0.0039	−0.734 ± 0.019	0.4226 ± 0.0831	4	3	−0.00978 ± 0.00128
myofibrillar, salt	0.575 ± 0.098	0.1178 ± 0.0039	−0.494 ± 0.110	0.2006 ± 0.0316	4	2	0.0 (not iterated)

[a] With $A_3 = K_3 = 0$ (two-step aggregation); an A_4C term was added to eq 25 for all data sets. [b] The association constant-related K_1 and K_2 are given in (g of protein/g of water)$^{-1}$. [c] There is no significant difference in the fitting parameters if data points corresponding to $\alpha_W > 0.95$ (i.e., solution samples) are not taken into account.

Table IV. Results of Nonlinear Regression Analysis of Water Sorption Data for Certain Proteins According to a Three-Step Protein Aggregation Model[a]

protein	parameters									
	A_1	K_1[b]	A_2	K_2[b]	A_3	K_3[b]	n	m	s	A_4
egg albumin	0.466 ± 0.019	0.1187 ± 0.0027	−0.085 ± 0.010	0.2773 ± 0.0117	−0.439 ± 0.017	0.0535 ± 0.0025	4	6	3	0.0 (NI)[c]
β-lacto-globulin	0.488 ± 0.007	0.1266 ± 0.0010	−0.102 ± 0.004	0.2929 ± 0.0043	−0.411 ± 0.008	0.0519 ± 0.0014	4	5	3	−0.00093 ± 0.00023
fibroin	0.423 ± 0.023	0.0824 ± 0.0013	−0.074 ± 0.009	0.2036 ± 0.0087	−0.387 ± 0.020	0.0381 ± 0.0029	4	5	3	−0.00015 ± 0.00050
gelatin	0.023 ± 0.060	0.1833 ± 0.0071	0.122 ± 0.035	0.2633 ± 0.0128	−0.015 ± 0.048	0.0609 ± 0.0241	3	10	3	0.0 (NI)
keratin	0.547 ± 0.051	0.1361 ± 0.0081	−0.136 ± 0.031	0.2362 ± 0.0082	−0.509 ± 0.026	0.0653 ± 0.0083	4	6	3	−0.00012 ± 0.00105
collagen	0.574 ± 0.033	0.1663 ± 0.0053	−0.210 ± 0.010	0.3656 ± 0.0053	−0.540 ± 0.031	0.0881 ± 0.0055	4	6	3	0.0 (NI)
salmin	0.695 ± 0.071	0.4334 ± 0.0603	−0.360 ± 0.062	0.1753 ± 0.0187	−0.659 ± 0.133	0.0587 ± 0.0019	2	3	6	−0.00161 ± 0.00627
myofibrillar	0.465 ± 0.150	0.1299 ± 0.0146	−0.289 ± 0.828	0.2617 ± 0.2611	−0.123 ± 0.193	0.4050 ± 0.1338	4	3	6	−0.00919 ± 0.00232
myofibrillar, salt	0.872 ± 0.021	0.5165 ± 0.0643	−0.227 ± 0.018	0.2322 ± 0.0051	−0.478 ± 0.066	0.1190 ± 0.0105	2	6	10	−0.01268 ± 0.00379

[a] Equation 25 with $A_3, K_3 \neq 0$; an additional A_4C term was also considered in all cases. [b] The association constant-related K_1, K_2, and K_3 are given in (g of protein/g of water)$^{-1}$. [c] Not iterated.

Table V. Assessment of the Goodness of Fit of Sorption Data According to Equations 19 and 24 with the Protein Aggregation Model

protein	SSR values[a]	
	two-step aggregation[b]	three-step aggregation[c]
egg albumin[d]	0.165×10^{-3}	0.532×10^{-4}
serum albumin	0.277×10^{-3}	
β-lactoglobulin		0.450×10^{-5}
zein B	0.203×10^{-3}	
zein C	0.178×10^{-3}	
fibroin		0.175×10^{-4}
gelatin[d]	0.789×10^{-3}	0.544×10^{-3}
elastin	0.123×10^{-3}	
salmin		0.125×10^{-3}
keratin		0.748×10^{-4}
collagen		0.285×10^{-4}
lysozyme	0.899×10^{-3}	
myofibrillar[e]	0.645×10^{-3}	0.626×10^{-3}
myofibrillar, salt[d]	0.682×10^{-3}	0.226×10^{-4}

[a] Sum of squares of the residuals, sum $[(\alpha_{w,exp} - \alpha_{w,calc})^2]$. [b] Obtained with parameters from Table III. [c] Obtained with parameters from Table IV. [d] The three-step model results in significantly better fittings. [e] There is no significant difference between the two fittings.

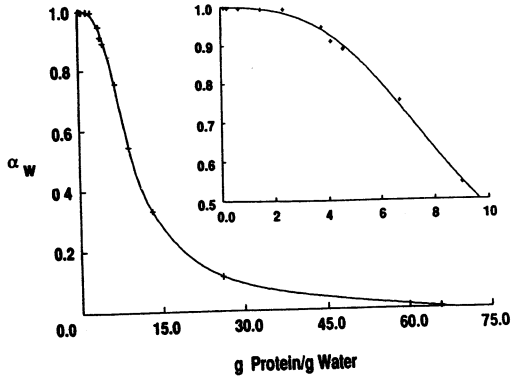

Figure 3. Lysozyme sorption data analyzed according to the two-step protein aggregation model. The isotherm was calculated from eq 18; the fitting parameters are given in Table III. The low protein concentration range (inset) can be described by the model reasonably well. The fitting parameters obtained when data points above $\alpha_w = 0.95$ are deleted do not differ significantly from the corresponding parameters of Table III.

concentrated lysozyme sample and the second aggregation step, (65.79 g of lysozyme/1 g of water at $\alpha_w \sim 0.005$) C_M = 99.2 mM and x = 663.45. In this case $k_2^m = 3.2 \times 10^{-22}$ and $mk_2^m[P_nW_q]^{m-1}[W] = 2.02 \times 10^{-26} \ll 1$ with $[P_nW_q]$ = 99.2/3 mM and $[W]$ = 1.2 M. Clearly, the approximations made are well justified.

Once again, it must be stressed that K_1, K_2, and K_3 (Tables III and IV) are merely parameters related to the equilibrium binding constants k_1, k_2, and k_3, respectively (see above); the various k's are average equilibrium constants for the association of protein monomers that yield an oligomer during the corresponding cooperative aggregation step.

The values of n, m, and s (Tables III and IV) represent the numbers of cooperatively associating protein molecules: initial formation of a n-mer from n protein monomers, followed by association of m n-mers at higher total protein concentration, and so on. The n, m, or s terms reflect only those protein aggregation processes that are linked to water activity changes; other types of occurring protein aggregation processes cannot be detected. The sensitivity of the RMS values to variations in the n, m, or s parameters (see Methods) was different for different proteins. Some showed a slow variation of RMS values over a wide range of n, m, or s, whereas for others the best n, m, and s value was more sharply defined.

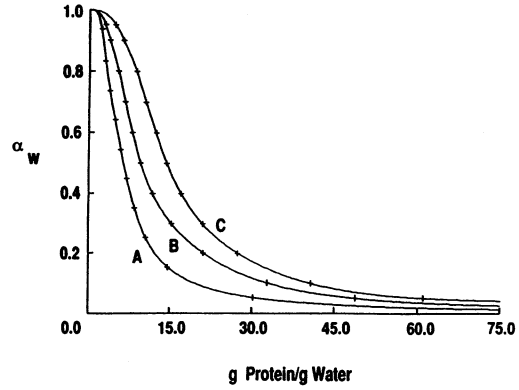

Figure 4. Experimental points and theoretical curves for collagen (A), β-lactoglobulin (B), and fibroin (C). A three-step protein aggregation model (eq 23) was employed. Fitting parameters are given in Table IV.

Table VI. Comparison of the Goodness of Fit of Protein Sorption Data between Our Protein Aggregation and the Isodesmic Model (D'Arcy and Watt, 1970, 1980)

	SSR_{norm} values[a]	
protein	aggregation model	isodesmic model[b]
egg albumin[d]	0.162×10^{-3}	0.0029
serum albumin[c]	0.515×10^{-3}	0.0233
β-lactoglobulin[d]	0.425×10^{-4}	0.0046
zein B[c]	0.127×10^{-4}	0.0076
zein C[c]	0.189×10^{-2}	0.0021
fibroin[d]	0.453×10^{-4}	0.0027
gelatin[d]	0.861×10^{-2}	0.0135
elastin[c]	0.715×10^{-2}	0.0199
salmin[d]	0.387×10^{-3}	0.0275
keratin[d]	0.162×10^{-2}	0.0030
collagen[d]	0.299×10^{-3}	0.0048
lysozyme[c]	0.143×10^{-1}	12.2818
myofibrillar[c]	0.120×10^{-2}	2.0406
myofibrillar, salt[d]	0.308×10^{-4}	1.5827

[a] Calculated as sum $[(\alpha_{w,exp} - \alpha_{w,calc})/\alpha_{w,exp}]^2$ for the protein aggregation model or as sum $[(M_{exp} - M_{calc})/M_{exp}]^2$ for the isodesmic model. [b] From eq 1; with parameters from Table I. [c] From eq 25 with $A_3 = K_3 = 0$ (two-step aggregation); with parameters from Table III. [d] From eq 25 (three-step aggregation); with parameters from Table IV.

6. CONCLUDING REMARKS

We have presented a plausible protein activity/aggregation model for the sorption of water vapor by proteins. The model provides very good agreement with experimental data over a wide range of water activities for a structurally diverse group of proteins with different hydration and solubility properties. Our model could be readily extended by adding terms for sequential or nonsequential aggregation, although we have not yet encountered a data set that required more than three aggregation steps. In addition to water sorption by proteins, hydration data for non-protein macromolecules (e.g., synthetic polymers such as polyacrylic acid and nylon or natural polymers such as cotton) can be successfully analyzed along the lines presented here (Kakalis and Kumosinski, unpublished results). [For thermodynamic treatments of both diffusion and protein activity effects, see also Tanford (1961).]

If the protein hydration is n_H (grams of bound water/gram of protein), then the fraction of bound water will be $(n_H\alpha_p)$, where α_p is the protein activity in grams of protein/gram of water (Kumosinski et al., 1988). Since α_w is a measure of the fraction of free water

$$\alpha_w + n_H\alpha_p = 1 \qquad (29)$$

Thus, for a given concentration C_p, one of α_w, n_H, or α_p may be obtained from eq 29 when the other two are known from independent measurements/calculations. The limited validity of this approach should be stressed. The α_w values thus obtained from NMR measurements of n_H and α_p agree with the relative vapor pressure (i.e., the α_w from sorption isotherms) only in the lower protein concentration range (Myers-Betts and Baianu, 1990). The deviation at higher protein concentrations is presumably due to the fact that NMR relaxation is sensitive to the fast, **short-range**, mostly rotational diffusion of water molecules either near the protein surface or in the protein interior, whereas water sorption isotherms are markedly affected by the presence of different textures, pores, or capillaries, possibly through capillary condensation of water vapor [eqs 6-42–6-48 in Baianu (1992)].

Hydration measurements in protein solutions have shown a decrease in the amount of bound water with increasing (electrostatic) protein association (Kakalis and Baianu, 1989), in agreement with the expected replacement of protein–water contacts by protein–protein ones. This finding does not contradict the predicted decrease in α_w with increasing protein aggregation since the effects of protein–protein interactions and protein aggregation on water associated with the aggregates more than compensates for some decrease in weak water binding at the protein surface, in agreement with the recent results of Lilley and Sutton (1991) for proline, sarcosine, and glycine–betaine, which showed a decreased α_w caused by solute–solute interactions even though apparent hydration numbers decreased.

The new approach to water sorption isotherms of proteins presented here links the reduction of water vapor pressure in concentrated protein suspensions and hydrated protein powders to protein aggregation/protein activity. Our approach emphasizes the importance of nonideal behavior in water sorption by a wide variety of proteins [see also Chapter 6 of Baianu (1992) for a detailed explanation of nonideal behavior]. Extension of our approach to include the effects of salts and humectants, in general, is now in progress and will be reported separately.

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APPENDIX

D'Arcy and Watt did not provide a detailed derivation of their model in their paper (D'Arcy and Watt, 1970) in terms of binding equilibria; such a derivation highlights the assumptions and limitations involved and is undertaken here.

We consider the case of a protein P that has n binding sites for a ligand L, water in our case, each with a macroscopic thermodynamic association constant k_i (Hammes, 1982). The binding equilibria are

$$\begin{aligned} P + L &\rightleftarrows PL_1 \quad & k_1 &= [PL_1]/[P][L] \\ PL_1 + L &\rightleftarrows PL_2 \quad & k_2 &= [PL_2]/[PL_1][L] \\ PL_{n-1} + L &\rightleftarrows PL_n \quad & k_n &= [PL_n]/[PL_{n-1}][L] \end{aligned} \qquad (A1)$$

The number of ν of ligand molecules bound per protein molecule is

$$\nu = \frac{[PL] + 2[PL_2] + ... + n[PL_n]}{[P] + [PL] + [PL_2] + ... + [PL_n]} \quad (A2)$$

or, taking into account eq A1

$$\nu = \frac{k_1[L] + 2k_1k_2[L]^2 + ... + nk_2k_2...k_n[L]^n}{1 + k_1[L] + k_1k_2[L]^2 + ... + k_1k_2...k_n[L]^n} \quad (A3)$$

This general equation can be simplified assuming that the binding sites are independent and identical, i.e., they all have the same intrinsic or microscopic association constant K. In relating macroscopic constants to the microscopic one, a correction for the statistical effect of the multiple binding sites must be applied: P has n sites that may be occupied by L, PL has $(n-1)$ such sites, and so on. The relationship between k_i and K is

$$k_i = (n - i + 1)/i\,K \quad (A4)$$

Substitution into eq A3 yields

$$\nu = \frac{nK[L] + \frac{2n(n-1)}{2!}K^2[L]^2 + ... + nk^n[L]^n}{1 + nk[L] + \frac{n(n-1)}{2!}K^2[L]^2 + ... + K^n[L]^n} \quad (A5)$$

or, from the binomial expansion

$$\nu = \frac{nK[L](1 + K[L])^{n-1}}{(1 + K[L])^n} = \frac{nK[L]}{1 + K[L]} \quad (A6)$$

If K and/or [L] are small enough so the $K[L] \ll 1$, then

$$\nu = nK[L] \quad (A7)$$

When the concept of binding is extended to multilayer formation and around n hydration sites, the statistical correction mentioned above is no longer necessary provided that the multilayer consists of an **infinite** number of hydration sites.

In this case

$$\nu = n\frac{[PL] + 2[PL_2] + 3[PL_3] + ...}{[P] + [PL] + [PL_2] + ...}$$

or, similarly to eqs A2 and A3

$$\nu = n\frac{K[P][L] + 2K^2[P][L] + ...}{[P] + K[P][L] + K^2[P][L]^2 + ...} \quad (A8)$$

After canceling out [P] (in this case because of partly hydrated protein without a multilayer) from eq A8, we note that the denominator is an infinite geometric progression and, assuming that $K[L] < 1$

$$1 + \sum_{i=1}^{\infty}(K[L])^i = 1 + \frac{K[L]}{1 - K[L]} = \frac{1.0}{1 - K[L]} \quad (A9)$$

The numerator summation can be written

$$\sum_{i=1}^{\infty} i(K[L])^i = K[L]\frac{d}{d(K)[L]}\sum_{i=1}^{\infty}(K[L])^i = K[L](1/(1 - K[L])^2) \quad (A10)$$

Substitution of eqs A9 and A10 into eq A8 yields

$$\nu = nK[L]/(1 - K[L]) \quad (A11)$$

If the hydration of a protein involves a combination of strong water binding to n_1 equivalent and independent protein sites and weak water binding to n_2 such sites, as well as multilayer formation around n_3 protein sites then, according to eqs A6, A7, and A11 for the total sorbed water

$$\nu = \frac{n_1K_1[L]}{1 + K_1[L]} + n_2K'[L] + \frac{n_3K_2[L]}{1 - K_2[L]} \quad (A12)$$

where K_1, K', and K_2 are the thermodynamic association constants that correspond to strong binding, weak binding, and multilayer formation, respectively. At thermodynamic equilibrium, the activity of free water [L] in eq A12 can be replaced by the partial pressure of water vapor (α_w) as a result of the equality of their chemical potentials. It is assumed that the contribution of bound water to the sample's water activity is negligible when compared to that of the free water. Also, ν can be related to the easily measured moisture content M of the sample (grams of water/gram of protein); the tacit assumption is that the amount of free water is negligible compared to the amount bound at the high protein concentrations studied. Then

$$M = \frac{18}{MW_p}\frac{n_1K_1\alpha_w}{1 + K_1\alpha_w} + \frac{18}{MW_p}n_2K'\alpha_w + \frac{18}{MW_p}\frac{mK_2\alpha_2}{1 - K_2\alpha_w} \quad (A13)$$

or, after the various constants are grouped together

$$M = \frac{K_1K_1'\alpha_w}{1 + K_1\alpha_w} + B\alpha_w + \frac{K_2K_2'\alpha_w}{1 - K_2\alpha_w} \quad (A14)$$

[an isodesmic model equivalent to that of D'Arcy and Watt (1970 and 1981)], with

$$K_1' = \frac{18n_1}{MW_p} \quad B = \frac{18n_2K'}{MW_p} \quad K_2' = \frac{18m}{MW_p} \quad (A15)$$

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